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Characterization of polyphenol oxidase activity in Ataulfo mango

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ABSTRACT

Crude extracts of Ataulfo exhibited polyphenol oxidase (PPO) activity with pyrogallol, 3-methylcatechol, catechol, gallic acid, and protocatechuic acid. The substrate dependent pH optima ranged from pH 5.4 to 6.4 with Michaelis–Menten constants between 0.84 ± 0.09 and 4.6 ± 0.7 mM measured in MES or phosphate buffers. The use of acetate buffers resulted in larger Michaelis–Menten constants, up to 14.62 ± 2.03 mM. Sodium ascorbate, glutathione, and kojic acid are promising inhibitors to prevent enzymatic browning in Ataulfo. PPO activity increased with ripeness and was always higher in the skin compared to the pulp. Sodium dodecyl sulphate (SDS) enhanced PPO activity, with pulp showing a stronger increase than skin. SDS–PAGE gels stained for catecholase activity showed multiple bands, with the most prominent bands at apparent molecular weights of 53, 112, and 144 kDa.

1. Introduction

Mango (*Mangifera indica*) is gaining importance as a commodity fruit in the Western Hemisphere. The consumption of mango in the United States, for example, has increased steadily from 0.2 kg per person in 1985 to 1.0 kg per person in 2010 (USDA, 2014). Mango is considered to be an excellent source of antioxidants, including phenolic compounds, carotenoids, and vitamin C (Ribeiro Queiroz, de Queiroz, Campos & Sant'Ana, 2007). Knight, Campbell, and Maguire (2009) describe over sixty economically important mango cultivars that differ in shape, colour, texture, and taste. The following cultivars are popular in the Western Hemisphere: Tommy Atkins, Haden, Kent, Keitt (all four developed in Florida), Francis from Haiti, and Ataulfo discovered in Mexico (Galán Saúco, 2010). According to Manthey and Perkins-Veazie (2009), the cultivar Ataulfo is unusual in its high antioxidant capacity. This mango variant, with its vibrant yellow colour, flattened oval shape, sweet taste, and string-less flesh, is already very popular in Mexico and is gaining consumers' interest in other countries (Galán Saúco, 2010). Notably, Mexico is the leading export country for mango, with 0.28 million tons reported for the export of mango in 2011. This data includes minor contributions from mangosteen and guava (FOASTAT, 2014). The cultivar Ataulfo is of great economic importance for the Chiapas region in Mexico (Hanemann, Bourns, & Fertziger, 2008).

Ataulfo and other mango are easily bruised upon harvesting and transportation. The bruising results in browning and limits the visual appeal and palpability of the fruit. The browning is initiated by polyphenol oxidase (PPO). This copper-containing enzyme catalyses the oxidation of phenolic compounds into highly reactive quinones which polymerize into dark coloured melanin (Yoruk & Marshall, 2003; Mayer, 2006). In some plants, PPOs also catalyse the hydroxylation of monophenols to ortho-diphenols (monophenolase or cresolase activity; EC 1.14.18.1) followed by the more common oxidation of ortho-diphenols to ortho-quinones (diphenolase or catecholase activity, EC 1.10.3.1). PPOs are involved in wound healing, pathogen defense, and several other cellular processes, such as control of oxygen levels in chloroplasts (Constabel, Bergey, & Ryan, 1995; Mayer, 2006).

The characterization of PPO activity in mango and other fruit is of great interest for the food and agricultural industry. Studies on the enzyme PPO extracted from mango pulp of the variety Tainong (Wang et al., 2007), of mango sap and skin of the Australian variety Kensington (Robinson, Loveys, & Chacko, 1993), and of the kernel from an African mango variety (Arogba, Ajiboye, Ugboko, Essienette, & Afolabi, 1998) have already been performed. The goal of our study was to characterize PPO activity in the skin and pulp of the mango variety Ataulfo. By selecting fruit in different stages of ripeness, we followed the change in PPO activity with fruit maturation. We also investigated substrate specificity, pH dependence, and inhibitor effectiveness to further our understanding of how enzymatic browning might be prevented in Ataulfo mango.

2. Materials and methods

2.1. Chemicals

Catechol, ethylenediaminetetraacetic acid (EDTA), gallic acid monohydrate, kojic acid, polyvinylpyrrolidone, protocatechuic

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acid, pyrogallol, sodium acetate, sodium azide, sodium dodecyl sulphate, and TRIS base were procured from Sigma–Aldrich (St. Louis, MO, USA). All other reagents, including 2-mercaptoethanol, 2-(N-morpholino)ethanesulphonic acid, 3-methylcatechol, citric acid, reduced glutathione, glycine, hydrochloric acid, sodium ascorbate, sodium benzoate, sodium chloride, sodium metabisulphite, monobasic sodium phosphate, dibasic sodium phosphate heptahydrate, glacial acetic acid, and N,N-diethyl-p-phenylenediamine sulphate, were obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Crude extract preparation

Ataulfo mangoes labelled with the PLU code #4312 from "Marathon – Produce of Mexico" were procured from a local supermarket in California. Fruit was stored at room temperature in a dry place. Crude extracts were prepared at different stages of ripeness as determined by visual and sensorial inspection. Based on the description of colour and firmness, our ripeness stages R1 to R4 are very similar to the stages RS1 to RS4 assigned by Palafox-Carlos, Yahia, Islas-Osuna, et al. (2012). The stage R1 is very green, immature, and hard to the touch. Stage R2 is green with yellow patches, but still hard and immature, whereas stages R3 and R4 are mature and slightly soft to the touch with increasing yellow colouration. Two over-ripe stages, R5 and R6, with increasing brown colouration were also included. Stage R6 is very soft to the touch. The skin and pulp of each fruit were separated from each other with a razor blade and further cut into smaller pieces before they were blended three times in 30 s intervals with an extraction buffer composed of 0.1 M sodium phosphate buffer, pH 6.8 with 1% w/v polyvinylpyrrolidone. The ratio of skin or pulp weight in gram to the volume of extraction buffer in millilitres was 1:5 with the exception of the sample R6-skin for which a 1:10 ratio was used, as this batch resulted in a particularly dense and thick extract. To screen for best assay conditions, another crude extract was prepared with an additional yellow, mature Ataulfo mango (stage R3) including its skin and pulp. All extracts were clarified by centrifugation for 30 min at 37,750 g and 4 °C. All samples were kept on ice or stored in small aliquots at −80 °C until further use.

2.3. Determination of protein content

Total protein content was determined using the Bradford method (Bradford, 1976). The Coomassie protein assay reagent and pre-diluted bovine gamma globulin standards from Pierce Biotechnology were procured from Thermo Scientific. Absorption measurements at 595 nm were performed with a Synergy H1 plate reader from Biotek. To validate the method for crude extracts from Ataulfo serial dilutions were prepared with samples R3-pulp, R4-pulp, R3-skin, and R4-skin. The Bradford assay yielded a linear response in the concentration range of 0.1–1.0 mg/mL total protein with correlation coefficients of 0.95 or higher. All samples were diluted with deionized water so that their protein content fell within this linear range.

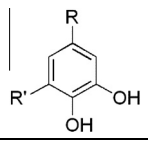
2.4. PPO activity measurements

The phenol oxidase activity was determined by monitoring the formation of coloured quinone products from various di- and triphenol substrates via time-dependent absorbance measurements using a Synergy H1 plate reader from Biotek. The temperature was set to 25 °C. A typical assay mixture contained 20 μL mango extract in a total reaction volume of 300 μL. The pH was controlled by using various buffers at 60 mM strength. Sodium acetate was used to cover the pH range 3.8–5.6. Sodium phosphate and 2-(N-morpholino)ethanesulphonic acid (MES) were employed for the pH ranges 5.6–7.8 and 5.2–6.6, respectively. Tris–HCl was used to cover the pH range 7.2–9.0. Substrate concentrations were varied between 0.2 and 30 mM to determine the enzyme kinetic parameter, K_M. Substrate dependent PPO activity curves were fit to the Michaelis–Menten equation using the program Enzfitter from Biosoft. In experiments to determine pH optima and substrate specificity or inhibitor effectiveness the final substrate concentration in the assay mixture was 30 mM. To determine the effectiveness, of various inhibitors, the PPO activity assays were conducted in 60 mM sodium phosphate buffer, pH 5.8 with 30 mM catechol and different concentrations of inhibitors in the range 20–0.02 mM. Two controls, without mango extract or without substrate, were subtracted from all main assays. Throughout the text, PPO activity values are given in International Units (abbreviated IU). One IU corresponds to the formation of one μmole quinone per minute. To express PPO activity in IU, Lambert Beer's law was used with molar absorptivity values (ε) determined individually for each quinone product. The measured slope (absorbance/min) was divided by the molar absorptivity value, ε, and the path length correction factor, l, of the plate reader followed by multiplication with the assay volume, V. The molar absorptivity values for each quinone product were determined by oxidizing the diphenolic substrates with a 20-fold excess of sodium periodate as described by Munoz et al. (2006). The slopes of these calibration curves yield the molar absorptivity values, ε (see Table 1).

2.5. Protein electrophoresis

Skin and pulp Ataulfo samples were prepared under partially denaturing conditions by mixing them with an equal volume of loading buffer composed of 1% w/v SDS, 20% v/v glycerol, and 100 mM TRIS, pH 6.8. The samples were not heated or reduced.

Table 1
PPO activity for diphenolic and triphenolic substrates with information on molar absorptivity of quinone-product and monitored wavelength.



Substituents R and R′	Wavelength (nm)	Molar absorptivity (M^{-1} cm^{-1})	pH-optimum	PPO activity (IU/mL)[a]
R=R′=H	420	1110[b] or 1225[c]	5.4–5.6	0.21 ± 0.02
R=H, R′=CH_3	400	1160[b] or 1420[c]	5.4–5.6	0.22 ± 0.01
R=H, R′=OH	320	3060[b,c]	5.8	0.50 ± 0.05
R=COOH, R′=H	420	1100[b]	6.2–6.4	0.059 ± 0.001
R=COOH, R′=OH	380	1610[b]	6.2–6.4	0.16 ± 0.01

[a] The mango extract had a total protein content of 2.42 ± 0.30 mg/mL and an extraction ratio of 0.2 g mango per one mL of extraction buffer. The PPO activity was determined with 30 mM substrate concentration. The value at the pH optimum is reported.
[b,c] The molar absorptivity value was determined in phosphate (b) or acetate (c) buffer, respectively.

Tris–glycine gels with a 4–20% acrylamide gradient and the See-Blue pre-stained protein standard were procured from Lifescience Technology. The gels were run in a cold room at 4 °C and 125 mV constant voltage for approximately 1.5 h in a running buffer composed of 3.02 g TRIS-base, 18.8 g glycine, and 1 g SDS per litre distilled water. The gels were stained for PPO activity according to the procedure of Rescigno, Sollai, Rinaldi, Soddu, and Sanjust (1997) with the exception of replacing the tertiary butyl-catechol reagent for catechol. Pictures of gels were recorded with a ChemiDoc MP Imaging System from BioRad. The determination of apparent molecular weights was performed with the software Image Lab from BioRad.

3. Results and discussion

3.1. Parameters that have an influence on PPO activity in crude Ataulfo mango extract

The most commonly used substrates to assess PPO activity in fruits or vegetables are phenolic compounds that derive from catechol. As summarized in Table 1, catechol, 3-methylcatechol, pyrogallol, protocatechuic acid, and gallic acid were employed in this study. Gallic acid is one of the most abundant phenolic compounds detected in mango fruit (Kim, Brecht, & Talcott, 2007; Palafox-Carlos, Yahia, González-Aguilar, 2012). The highest PPO activity was found for the tri-phenolic compound pyrogallol. Catechol and 3-methylcatechol showed similar reactivity. The presence of an additional hydroxyl group, as found in pyrogallol, therefore enhances PPO activity in contrast to a methyl group or a hydrogen atom in the same position. Substrates carrying a carboxylic acid group displayed the lowest PPO activity. Gallic acid, a tri-phenolic compound, is a superior substrate compared to protocatechuic acid, a di-phenolic compound.

The pH profiles for the PPO catalysed oxidations of catechol, 3-methylcatechol, pyrogallol, protocatechuic acid, and gallic acid are shown in Fig. 1. Catechol and 3-methylcatechol showed the same pH optimum at 5.4–5.6. The presence of an additional hydroxyl group and/or a carboxylic acid group raised the pH optimum of the PPO-catalysed reaction. At pH-values above 7.0, control assays with the triphenolic compounds, pyrogallol and gallic acid, showed a high auto-oxidation rate in the absence of mango extract, approaching and almost exceeding the rate of the enzyme catalysed reaction. For all other conditions, control assays showed rates that were two orders of magnitude smaller than the main catalysed reaction. Mango pulp is known to be acidic with pH values at or below pH 4 (Mannan, Khan, Islam, Islam, & Siddiqa, 2003; Tovar, García, & Mata, 2001). The major organic acids present in the pulp of mangoes are citric acid and malic acid (Medlicott & Thompson, 1985). At such low pH values, none of the tested substrates exhibited significant PPO activity. Also, as long as the cell organelles are still intact, most of the phenolic compounds will be enclosed in a vacuole separate from the sub-cellular location of PPO (Toivonen & Brummell, 2008). PPO initiated browning with internal substrates, such as gallic acid, only becomes important when the integrity of cellular compartments is destroyed, oxygen is available, and the local pH is not too acidic.

PPO activity assays with varied substrate concentrations exhibited a typical Michaelis–Menten profile in the concentration range of 0.2–30 mM. Higher substrate concentrations often resulted in the formation of opaque solutions or precipitation. This might be due to subsequent reactions involving the highly reactive quinone products formed in the activity assay. The Michaelis–Menten parameters are 1.25 ± 0.14 mM, 1.31 ± 0.18 mM, 0.84 ± 0.09 mM, 4.6 ± 0.7 mM, for the substrates catechol, 3-methylcatechol, pyrogallol, and gallic acid, determined at their respective optimum pH-value using MES or sodium phosphate buffers.

Michaelis–Menten profiles were recorded for the substrates catechol, 3-methylcatechol, and pyrogallol at different pH values in acetate, MES, and sodium phosphate buffers. The enzyme kinetic parameters are summarized in Table 2. All data recorded with acetate buffers exhibit a pronounced shift to larger K_M values. Compounds with carboxylic acid groups, such as citric acid and malic acid, have been shown to inhibit PPO in other fruit (Yoruk & Marshall, 2003). Our screen for PPO inhibitors (see Table 3) also showed that citric acid and, to a much lesser extent, benzoic acid are potential agents to prevent enzymatic browning of Ataulfo. This inhibitory effect might simply be due to the lowering of the

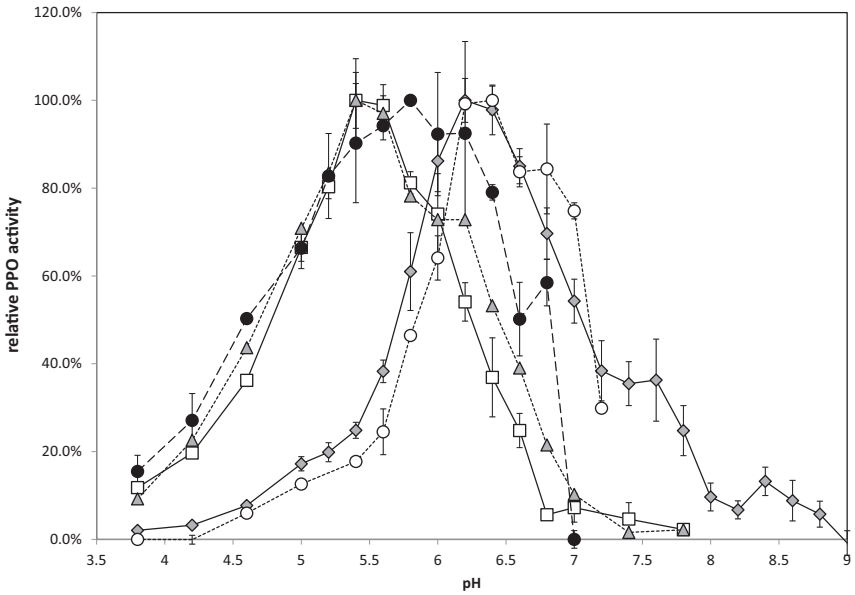


Fig. 1. Dependence of PPO activity on pH-value for the substrates catechol (white squares), 3-methylcatechol (grey triangles), pyrogallol (black circles), protocatechuic acid (grey diamonds), and gallic acid (white circles). For each substrate the relative activity values were normalized with the PPO activity values given in Table 1. Measurements were performed in triplicate. The graph depicts the average value with error bars of one standard deviation in both directions.

Table 2
Enzyme kinetic parameters (K_M and v_{max}) obtained by fitting substrate variation curves.

Substrate	Buffer	K_M (mM)	v_{max} (IU/mL)
Catechol	Sodium acetate, pH 5.4	14.62 ± 2.03[a]	0.28 ± 0.02[b]
	Sodium acetate, pH 5.6	10.59 ± 0.78	0.29 ± 0.01
	MES, pH 5.6	1.25 ± 0.14	0.24 ± 0.01
	MES, pH 5.8	1.07 ± 0.11	0.22 ± 0.01
	MES, pH 6.0	0.68 ± 0.16	0.19 ± 0.01
	Sodium phosphate, pH 5.8	0.87 ± 0.15	0.20 ± 0.01
	Sodium phosphate, pH 6.0	0.63 ± 0.20	0.19 ± 0.01
	Sodium phosphate, pH 6.2	0.41 ± 0.09	0.17 ± 0.01
3-Methylcatechol	Sodium acetate, pH 5.4	3.69 ± 0.51	0.24 ± 0.02
	MES, pH 5.6	1.31 ± 0.18	0.26 ± 0.02
Pyrogallol	Sodium acetate, pH 5.6	7.31 ± 0.77	0.53 ± 0.02
	MES, pH 5.4	1.26 ± 0.32	0.48 ± 0.03
	MES, pH 5.8	0.84 ± 0.09	0.46 ± 0.01
	MES, pH 6.0	0.79 ± 0.09	0.43 ± 0.01
	Sodium phosphate, pH 5.8	0.83 ± 0.09	0.49 ± 0.01

[a] Data is presented as value of the fit ± error of the fit.
[b] The unit conversion for v_{max} into IU/mL was carried out as described in the experimental section.

Table 3
Effect of various inhibitors on PPO activity in crude extracts of Ataulfo.

Inhibitor	20 mM	2 mM	0.2 mM	0.02 mM
β-Mercaptoethanol	0%[a]	0%	0%	0%
Sodium metabisulphite	0%	0%	0%	24%
Sodium azide	0%	0%	1%	47%
Sodium ascorbate	0%	0%	6%	93%
Glutathione	0%	0%	6%	102%
Kojic acid	0%	4%	40%	96%
Citric acid	0%	53%	88%	101%
EDTA	23%	27%	69%	100%
Sodium chloride	21%	46%	81%	102%
Benzoic acid	44%	101%	103%	97%

[a] 100% relative PPO activity corresponds to reaction rates that are identical to a reference condition without inhibitor. Measurements were performed in duplicate or triplicate with standard deviations ranging from 2% to 16%.

pH value (acidulating agents) or a metal-chelating effect for inhibitors with multiple carboxylic acid groups. It is still puzzling, however, that the presence of acetate buffer with only one carboxylate group raises the apparent K_M values in comparison to other buffers used to stabilize the same pH value.

To investigate the temperature stability of PPO, crude Ataulfo extracts were placed for a duration of either 10, 20, 30, 40, 50 and 60 min into a water bath set to either 30, 50, or 70 °C. The crude extracts were cooled in an ice water bath and let recover to room temperature before resuming with PPO activity measurements. More than 30 min at 70 °C were necessary to reduce the PPO activity by 50%. Polyphenol oxidase activity in crude extracts prepared from the skin of the mango variety Kensington also revealed high thermostability (Robinson et al., 1993). Heating is therefore not a valid treatment to prevent the browning of Ataulfo and Kensington mango cultivars.

3.2. Inhibition of PPO activity in crude extracts of Ataulfo mango

Several compounds that were shown to inhibit PPO in other fruit (Yoruk & Marshall, 2003) were tested for their effectiveness to prevent enzymatic browning in crude extracts of Ataulfo mango. According to the data shown in Table 3, the thiol-containing compound beta-mercaptoethanol and the copper binding ligand azide are highly effective, but also very toxic. Sodium metabisulphite is also very effective, but this compound was banned as a food additive by the FDA in 1995 (Martinez & Whitaker, 1995). Commercially available anti-browning mixtures often contain ascorbic and citric acid (Loizzo, Tundis, & Menichini, 2012). Sodium ascorbate was a more effective inhibitor compared to citric acid. Synergistic effects were not investigated in this study. Glutathione (another thiol-containing compound) and kojic acid show potential as useful inhibitors. Inhibition of PPO activity was also observed for EDTA, sodium chloride, and benzoic acid albeit at higher concentrations compared to any of the other compounds.

3.3. Dependence of PPO activity on the maturity in the skin versus the pulp of Ataulfo

Mangoes in ripeness stages R1–R6 were separated into skin and pulp samples. All samples (see Table 4) showed higher PPO activity per gram skin than per gram pulp. These differences ranged from 5-fold to 25-fold. Sample R1 was an exception with only a 2-fold difference between the PPO activities of pulp and skin. This sample from the most green and unripe Ataulfo also showed the lowest protein content for pulp and skin, and the sample R1-pulp exhibited a surprisingly high activity of 0.60 ± 0.05 IU/g. All other samples tend to show an increase in activity with maturation. The most overripe Ataulfo (R6) clearly displayed the highest PPO activities in pulp and skin.

In agreement with our observations, Robinson et al. (1993) also observed a significant difference in PPO activity for skin and pulp samples of a ripe mango from the variety Kensington with 25-fold higher activity for the skin compared to the pulp sample. No PPO activity was detected in the pulp of unripe Kensington. The skin of unripe Kensington displayed a 2.5-fold reduced activity compared to the ripe Kensington skin sample (Robinson et al., 1993).

Table 4
PPO activity and protein content in dependence of ripeness stage of pulp and skin Ataulfo samples.

Sample	Ripeness stage	Protein content (mg/g)[a]	PPO activity (IU/g)[a]	PPO activity with 1% SDS (IU/g)[a]	SDS enhancement
Pulp	R1	2.8 ± 0.7	0.60 ± 0.05	8.99 ± 0.82	15
	R2	10.6 ± 0.4	0.22 ± 0.06	8.00 ± 2.34	36
	R3	20.6 ± 4.5	0.19 ± 0.05	11.06 ± 1.03	58
	R4	21.4 ± 1.8	0.56 ± 0.13	13.01 ± 2.66	23
	R5	10.7 ± 1.3	0.85 ± 0.20	20.74 ± 5.98	24
	R6	16.7 ± 2.6	2.70 ± 1.71	20.65 ± 4.27	8
Skin	R1	3.1 ± 0.3	1.12 ± 0.16	5.25 ± 0.49	5
	R2	9.2 ± 0.9	3.49 ± 0.96	12.62 ± 3.64	4
	R3	9.4 ± 0.7	4.71 ± 0.53	10.69 ± 0.70	2
	R4	13.2 ± 0.3	8.26 ± 1.91	12.63 ± 1.76	2
	R5	11.5 ± 0.6	7.23 ± 0.97	20.45 ± 2.05	3
	R6	7.7 ± 0.9	14.78 ± 1.24	67.90 ± 14.77	5

[a] Protein content and PPO activity are reported per gram mango (skin or pulp). All measurements were performed in triplicate. Data is presented as mean ± standard deviation.

As fruit ripen and soften, several biochemical processes change (Giovannoni, 2001). It is not uncommon for fruit to show higher activity for specific enzymes in more mature stages. For example, the activity of the enzyme β-galactosidase increases during ripening of mango (Ali, Armugam, & Lazan, 1995). Antioxidant capacity, quantity of phenolic compounds, and respiration were also shown to increase during the first three stages of Ataulfo maturation followed by a slight decrease in the later stages of ripeness (Palafox-Carlos, Yahia, Islas-Osuna, et al., 2012).

Many researchers observed that PPO activity can be enhanced by adding the anionic detergent SDS. The degree of SDS activation varies greatly with plant material and experimental conditions. In their review, Yoruk and Marshall (2003) present a range of 4-fold to 119-fold increased PPO activity upon SDS addition. It is conceivable that PPO is present in a latent form with a regulatory domain blocking the catalytic site of PPO which becomes more accessible after SDS addition, acidification, or proteolytic treatment (Yoruk & Marshall, 2003). Notably, our data in Table 4 showed much higher SDS activation for pulp compared to skin samples. In fact, with an addition of 1% w/v SDS, most pulp samples approach or exceed the activity values of skin samples at the same ripeness stage. We tentatively suggest a difference in latency for PPO in the pulp versus the skin of Ataulfo.

3.4. Apparent molecular weight of bands with PPO activity in partially denaturing SDS–PAGE

SDS–PAGE gels stained for PPO activity with the substrate catechol are presented in Fig. 2. With the exception of R1, all pulp and skin samples show a band with an apparent molecular weight of approximately 53 kDa. Sample R1-pulp which exhibited an unusually high PPO activity value for a pulp sample of an unripe, green mango, display a band at approximately 144 kDa that is also found in all skin samples. The two brown pulp samples (R5 and R6) also show a band located at 112 kDa. Less prominently stained bands are located at 23 kDa (R2-pulp) and 72 kDa (R5-pulp).

Multiple forms of PPO differing in their electrophoretic mobility have been observed in a large variety of plants with molecular weights ranging from 32 kDa to over 200 kDa, with most molecular weights between 35 and 70 kDa (Yoruk & Marshall, 2003). Possible reasons for this multiplicity are the attachment of phenolic oxidation products or carbohydrates, proteolysis, conformational changes, oligomerization, and finally the presence of distinctly different genes. Information on PPO genes from mango is limited to the deposition of one partial mRNA coding sequence from *Mangifera indica*, Linn (GenBank: GU266283.1). Mango belongs to the subclass Rosidae (Malvids). Within this subclass, four full-length mRNA coding sequences are available yielding calculated molecular weights of 68.5 kDa for the *Canarium album* cultivar Huiyuan (GenBank: JQ319005.1), 67.2 kDa for *Gossypium hirsutum* (GenBank: JQ345705.1), 69.5 kDa for *G. hirsutum* clone ZS1 (GenBank: JX966316.1), and 66.2 kDa for *Citrus clementina* (NCBI Reference Sequence: XM_006449228.1). Since the samples applied to the SDS–PAGE gels in Fig. 2 are only partially denatured, the band positions indicate only apparent molecular weights. It is conceivable that the bands located at 53 or 72 kDa represent the main PPO isoform either with or without a proteolytic cut. Robinson and coworkers (Mazzafera & Robinson, 2000; Robinson & Dry, 1992) demonstrated that PPO from coffee and broad bean leaves can undergo proteolysis without loss of catalytic capacity as the apparent molecular weight is reduced from 67 to 45 kDa or 60–42 kDa, respectively. The bands located at higher molecular weights of 112 and 144 kDa might represent dimeric forms, but the presence of artifacts due to attachment of polyphenol oxidation products cannot be ruled out. The higher molecular weight bands were only apparent in samples with PPO activity values above 0.60 ± 0.05 IU per gram mango. The higher the intrinsic activity is, the more likely the attachment of polyphenol oxidation products. Further work on purified PPO from Ataulfo and a full nucleotide sequence determination will be necessary to clarify the origin of the multiple PPO forms.

4. Conclusion

PPO activity in crude extracts of Ataulfo mango was observed for di- and tri-phenolic substrates with pH optima between pH 5.4 and pH 6.4. The enzyme was fairly thermo-stable, but can be inhibited effectively with sodium ascorbate in millimolar concentrations. L-Ascorbic acid is listed as a GRAS compound by the FDA (FDA, 2014). GRAS stands for "Generally Recognized As Safe".

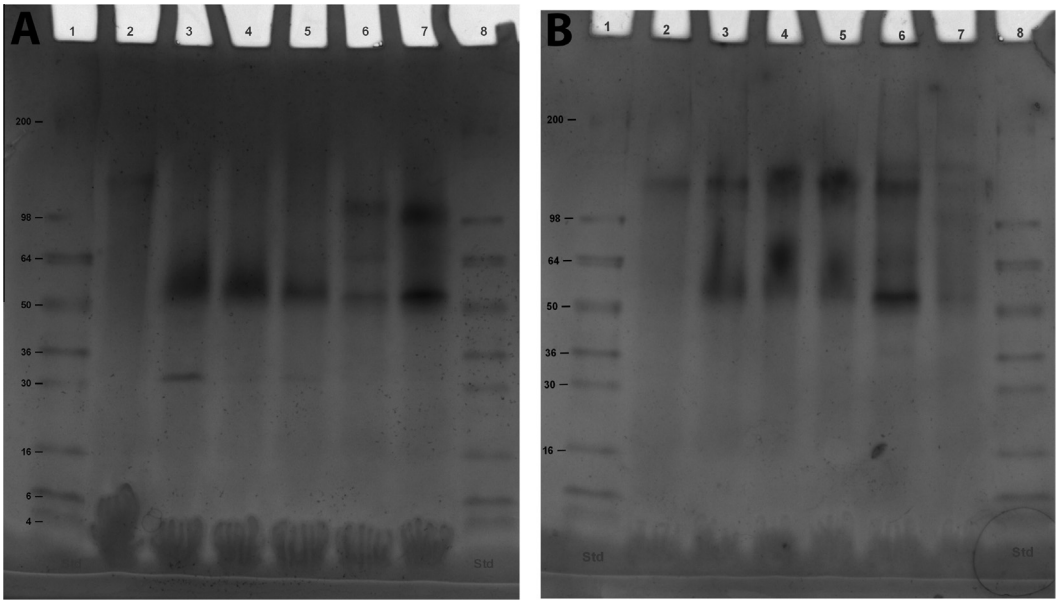


Fig. 2. Partially denaturing 4–20% Tris–glycine SDS–PAGE gels stained for PPO activity. Ataulfo pulp (A) and skin (B) samples with increasing ripeness (stages R1-R6) were loaded into the lanes 2–7. Lanes 1 and 8 contain the pre-stained protein standard SeeBlue from Life Technologies.

PPO activity as well as PPO isoform distribution depended on ripeness stage and part of the fruit. The highest PPO activity was found in skin samples of very overripe Ataulfo. Samples with high PPO activity displayed at least two PPO isoforms (53 and 112 or 144 kDa), whereas samples with low PPO activity showed only one major band at 53 kDa in partially denaturing SDS–PAGE gels stained for catecholase activity. The anionic detergent SDS was an activator of PPO. SDS enhancement was notably stronger for pulp compared to skin samples. The ripeness stage and the part of the plant that was used for extract preparation should therefore always be considered in the evaluation or comparison of enzymatic browning reactions.

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